

PATENT APPLICATION

**CYTOKINE USES; COMPOSITIONS; METHODS**

INVENTORS:

Stephen D. Hurst, a citizen of the United States, residing at 2501 Harrison Street, #8;  
San Francisco, California 94110;

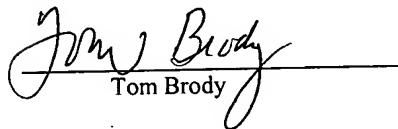
Sandra M. Zurawski, a citizen of the United States, residing at 2005 School Road; San  
Juan Bautista, California 95045; and

Donna M. Rennick, a citizen of the United States, residing at 740 Vista Grande, Los  
Altos, California 94024.

"Express Mailing" mailing label number EL 982 851 029 US

Date of Deposit November 10, 2003

I hereby certify that this paper is being deposited with the United States Postal Service  
"Express Mail Post to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above  
and is addressed to: Mail Stop Patent Application; Commissioner for Patents; Washington  
D.C. 22313-1450.

Tom Brody Nov. 10, 2003  
date

*Customer No. 28008*

DNAX Research, Inc.  
901 California Avenue  
Palo Alto, California 94304-1104  
Tel: (650) 496-6400  
Fax: (650) 496-1200

## CYTOKINE USES; COMPOSITIONS; METHODS

This application claims benefit of U.S. provisional patent application  
5 no. 60/198,488, filed April 18, 2000.

## Field of the Invention

The present invention relates to compositions related to proteins which function in controlling physiology, development, and differentiation of mammalian cells, e.g., cells of a 10 mammalian immune system. In particular, it provides nucleic acids, proteins, antibodies, and mimetics which regulate cellular physiology, development, differentiation, or function of various cell types, including hematopoietic cells.

## BACKGROUND OF THE INVENTION

15 The immune system of vertebrates consists of a number of organs and several different cell types. Two major cell types include the myeloid and lymphoid lineages. Among the lymphoid cell lineage are B cells, which were originally characterized as differentiating in fetal liver or adult bone marrow, and T cells, which were originally characterized as differentiating in the thymus. See, e.g., Paul (ed. 1998) Fundamental  
20 Immunology (4th ed.) Raven Press, New York.

In many aspects of the development of an immune response or cellular 25 differentiation, soluble proteins known as cytokines play a critical role in regulating cellular interactions. These cytokines apparently mediate cellular activities in many ways. They have been shown, in many cases, to modulate proliferation, growth, and differentiation of hematopoietic stem cells into the vast number of progenitors composing the lineages responsible for an immune response.

However, the cellular molecules which are expressed by different developmental 30 stages of cells in these maturation pathways are still incompletely identified. Moreover, the roles and mechanisms of action of signaling molecules which induce, sustain, or modulate the various physiological, developmental, or proliferative states of these cells is poorly understood. Clearly, the immune system and its response to various stresses had relevance to medicine, e.g., infectious diseases, cancer related responses and treatment; allergic and transplantation rejection responses. See, e.g., Thorn, et al. Harrison's Principles of Internal Medicine McGraw/Hill, New York.

35 Medical science relies, in large degree, on appropriate recruitment or suppression of the immune system in effecting cures for insufficient or improper physiological responses to environmental factors. However, the lack of understanding of how the immune system is

regulated or differentiates has blocked the ability to advantageously modulate the normal defensive mechanisms to biological challenges. Medical conditions characterized by abnormal or inappropriate regulation of the development or physiology of relevant cells thus remain unmanageable. The discovery and characterization of specific cytokines will 5 contribute to the development of therapies for a broad range of degenerative or other conditions which affect the immune system, hematopoietic cells, as well as other cell types. The present invention provides solutions to some of these and many other problems.

#### SUMMARY OF THE INVENTION

10 The present invention is based, in part, upon biological activities of the cytokine known as IL-174. In particular, a number of assays indicate that this cytokine has functions in regulating establishment of Th2 type immune responses, innate immunity, inflammatory responses, mucosal and fibroblast growth, certain hematopoietic activities, and granuloma formation.

15 The present invention provides methods: directing a mammalian immune response towards a Th2 type response, the method comprising administering an IL-174 agonist to immune cells of the mammal; stimulating an mammalian innate immune response, the method comprising administering an IL-174 agonist to immune cells of the mammal; augmenting a mammalian inflammatory response from epithelial or fibroblast cells, the 20 method comprising further administering an IL-174 agonist to the mammal; inducing gut cell growth, the method comprising administering an IL-174 agonist to the cell; promoting mammalian extra medulary hematopoiesis, the method comprising administering an IL-174 agonist to the mammal; or augmenting antibody responses in serum and fecal material, comprising administering an IL-174 agonist to the cell.

25 In various embodiments, the method involves administering an agonist, wherein the administering: induces cytokine production by a hematopoietic, fibroblast, epithelial, or endothelial cell; downregulates an inflammatory response which accompanies an infection; stimulates growth of an epithelial cell; or induces growth of gut epithelial, fibroblast, or goblet cells. In some embodiments, the method involves administering an agonist, wherein 30 the mammal exhibits, or has experienced conditions to stimulate: an autoimmune condition; an infectious disease immune response; a wound healing response; or a Th1 mediated condition. In further embodiments, the autoimmune condition is selected from: multiple sclerosis, systemic lupus erythematosis, rheumatoid arthritis, diabetes, or psoriasis; the infectious response is symptomatic of: an Aspergillus infection, a fungal infection 35 (including Candidiasis, Blastomycosis, or Aspergillosis), a parasitic infection (including Schistosomiasis, fluke worm, Helminth, or Filariasis); or a viral infection (including

hepatitis); or the Th1 mediated condition is an inflammatory condition (including Crohn's disease, ulcerative colitis, pancreatitis, or hepatitis). Additionally, the invention provides methods treating an infectious response, further comprising administering another therapeutic entity to treat the infection.

5 The invention also provides other methods, e.g., directing a mammalian immune response away from a Th2 type response, the method comprising administering an IL-174 antagonist to immune cells of the mammal; or preventing mammalian inflammation or granuloma formation, comprising administering an IL-174 antagonist to immune system cells. Often, the antagonist is a monoclonal or polyclonal antibody against IL-174. The 10 method may involve administering an antagonist, wherein: the administering blocks eosinophil attraction, tissue remodeling, or fibrosis; or the mammal exhibits, or has experienced conditions to stimulate: an allergic condition; an inflammatory condition; or a Th2 mediated condition. In further embodiments, the eosinophils are attracted to the lung (i.e., asthma), liver or intestine (i.e., eosinophilic gastritis); the fibrosis is pancreatic duct or 15 peribiliary fibrosis; the antagonist suppresses production of IL-4, IL-5, and/or IL-13; the antagonist decreases eotaxin, CCR4, and/or CCR4 expression in BAL; symptoms of the allergic condition are in the lung; the allergic condition is a systemic anaphylactic response, skin hypersensitivity response, or a food allergy; or the inflammatory or Th2 mediated condition is a dermatitis or asthmatic inflammation. In yet other embodiments, the mammal 20 exhibits, or has experienced conditions to stimulate: an allergic condition; an inflammatory condition; or a Th2 mediated condition.

25 The invention also provides a composition comprising: an IL-174 agonist and: an antimicrobial (including an antibiotic, antiviral, or antifungal compound) or a chemotherapy agent; or an IL-174 antagonist and: an allergy medicament, an asthma medicament, a dermatitis medicament, a fibrosis medicament, or eosinophilic gastritis medicament.

## DETAILED DESCRIPTION OF THE INVENTION

### Outline

- 30 I. General
- II. Cytokine Agonists and Antagonists
  - A. IL-174 and Variants
  - B. Antibodies
  - C. Other Molecules
- 35 III. Immunoassays
- IV. Uses

## I. General

The invention is based, in part, on the surprising discovery that the cytokine designated IL-174 has roles in various aspects of immune responses. The IL-174 is one of a family of genes encoding proteins which exhibit structural features characteristic of cytokines, particularly related to the cytokine designated CTLA-8 (also referred to as IL-17). Rat, mouse, human forms and a viral homolog of CTLA-8 have been described and their sequences available from GenBank. See Rouvier, et al. (1993) *J. Immunol.* 150:5445-5456; Yao, et al. (1995) *Immunity* 3:811-821; Yao, et al. (1995) *J. Immunol.* 155:5483-5486; and Kennedy, et al. (1996) *J. Interferon and Cytokine Res.* 16:611-617. The CTLA-8 has activities implicated in arthritis, kidney graft rejection, tumorigenicity, virus-host interactions, and innate immunity; and appears to exhibit certain regulatory functions similar to IL-6. See PubMed (search for IL-17); Chabaud, et al. (1998) *J. Immunol.* 63:139-148; Amin, et al. (1998) *Curr. Opin. Rheumatol.* 10:263-268; Van Kooten, et al. (1998) *J. Am. Soc. Nephrol.* 9:1526-1534; Fossiez, et al. (1998) *Int. Rev. Immunol.* 16:541-551; Knappe, et al. (1998) *J. Virol.* 72:5797-5801; Seow (1998) *Vet. Immunol. Immunopathol.* 63:139-48; and Teunissen, et al. (1998) *J. Invest. Dermatol.* 111:645-649. A report on the signaling through the NF $\kappa$ B transcription factor implicates a signal pathway which is used in innate immunity. Shalom-Barak, et al. (1998) *J. Biol. Chem.* 273:27467-27473.

The IL-174 cDNA sequences exhibit various features which are characteristic of mRNAs encoding cytokines, growth factors, and oncogenes. The IL-17 is the first member of this newly recognized family of cytokines related to TGF- $\beta$ , and a number of members of this family designated "IL-170" have been identified. The fold for this family is predicted to be that of the TGF- $\beta$  family of cytokines. The TGF- $\beta$  family of cytokines, and the IL-170 family share the common feature of a cystine knot motif, characterized by a particular spacing of cysteine residues. See, e.g., Sun and Davies (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:269-291; McDonald, et al. (1993) *Cell* 73:421-424; and Isaacs (1995) *Curr. Op. Struct. Biol.* 5:391-395. In particular, the structures suggest a number of conserved cysteines. The disulfide linkages should be cysteines 2 with 5; and 3 with 6; and 1 with 4. Functional significance of the fold similarity suggests formation of dimers for the IL-170 family. As a consequence, IL-170 dimers would bring together two cell surface receptors, through which signal transduction will occur.

These new proteins are designated CTLA-8 related, or generally IL-170, proteins. The natural proteins should be capable of mediating various physiological responses which would lead to biological or physiological responses in target cells, e.g., those responses characteristic of cytokine signaling.

Purified CTLA-8, when cultured with synoviocytes, is able to induce the secretion of IL-6 from these cells. This induction is reversed upon the addition of a neutralizing antibody raised against human CTLA-8. Endothelial, epithelial, fibroblast and carcinoma cells also exhibit responses to treatment with CTLA-8. This data suggests that CTLA-8 5 may be implicated in inflammatory fibrosis, e.g., psoriasis, scleroderma, lung fibrosis, or cirrhosis. CTLA-8 may also cause proliferation of carcinomas or other cancer cells inasmuch as IL-6 often acts as a growth factor for such cells. As such, the newly discovered other related family members are likely to have similar or related biological activities.

10 The cytokine IL-174, from mouse and human, has been described earlier in PCT/US00/00006, which is incorporated herein by reference for all purposes. The nucleic acid sequence and the corresponding amino acid sequence encoding human IL-174 are set forth as SEQ ID Nos. 1 and 2 respectively. Important predicted structural motifs include, e.g., cAMP PK sites at 21-24, 53-56, and 95-98; Ca phosphorylation sites at 15-17, 16-18, and 45-47; myristoly sites at 12-16, 115-119, and 118-122; N-glycosyl site at 104-107; 15 phosphorylation sites at 21, 23, 43, 53, 56, 95, 98, and 131; PKC phosphorylation sites at 41-43 and 119-121; and tyrosine kinase site at 95-102. The mouse nucleic acid and corresponding amino acid sequence encoding IL-174 are set forth as SEQ ID Nos. 3 and 4 respectively. Important predicted motifs include, e.g., cAMP PK sites at 29-32 and 61-64; 20 Ca phosphorylation sites at 18-20, 53-55, and 67-69; myristoly site at 123-127; N-glycosylation site at 112-114; and phosphorylation sites at 29, 31, 51, 53, 61, 64, 139, and 141; and PKC phosphorylation sites at 2-4, 49-51, and 127-129.

## II. Cytokine Agonists and Antagonists

25 Mammalian IL-174 cytokines were described previously in PCT/US00/00006. Various agonists and antagonists of the natural ligands can be produced.

### A. IL-174 and variants

IL-174 agonists will exhibit some or all of the signaling functions of the cytokine. Various mammalian IL-174 sequences may be evaluated to determine what residues are 30 conserved across species, suggesting what residues may be changed without dramatic effects on biological activity. Alternatively, conservative substitutions are likely to retain biological activity, thus leading to variant forms of the cytokine which will retain agonist activity. Standard methods for screening mutant or variant IL-174 polypeptides will determine what sequences will be useful therapeutic agonists.

35 In addition, certain nucleic acid expression methods may be applied. Various promoters may be operably linked to the gene, thereby allowing for regulated expression.

Alternatively, antagonist activity may be tested for. Tests for ability to antagonize cytokine activity can be developed using assays as described below. Various ligand homologs can be created which retain receptor binding capacity, but lacking signaling capability can be prepared. Small molecules may also be screened for ability to antagonize 5 IL-174 function. See generally Gilman, et al. (eds. 1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn, each of which is incorporated herein by reference.

10 B. Antibodies  
The present invention provides for the use of an antibody or binding composition which specifically binds to IL-174, preferably mammalian, e.g., primate, human, cat, dog, rat, or mouse, and neutralizes the ability of the cytokine to mediate its signal. Antibodies can be raised to various IL-174 proteins, including individual, polymorphic, allelic, strain, or species variants, and fragments thereof, both in their naturally occurring (full-length) 15 forms or in their recombinant forms. Additionally, antibodies can be raised to IL-174 polypeptides in both their native (or active) forms or in their inactive, e.g., denatured, forms, which may neutralize ligand capacity to mediate its signal. Antibodies may block the interaction of the ligand with its receptor.

20 A number of immunogens may be selected to produce antibodies specifically reactive, or selective for binding, with IL-174 proteins. Recombinant protein is a preferred immunogen for the production of monoclonal or polyclonal antibodies. Naturally occurring protein, from appropriate sources, e.g., primate, rodent, etc., may also be used either in pure or impure form. Synthetic peptides, made using the IL-174 protein sequences described herein, may also be used as an immunogen for the production of antibodies to IL-174 25 proteins. Recombinant protein can be expressed and purified in eukaryotic or prokaryotic cells as described, e.g., in Coligan, et al. (eds. 1995 and periodic supplements) Current Protocols in Protein Science John Wiley & Sons, New York, NY; and Ausubel, et al (eds. 1987 and periodic supplements) Current Protocols in Molecular Biology, Greene/Wiley, New York, NY. Naturally folded or denatured material can be used, as appropriate, for 30 producing antibodies. Either monoclonal or polyclonal antibodies may be generated, e.g., for subsequent use in immunoassays to measure the protein, or for immunopurification methods.

35 Methods of producing polyclonal antibodies are well known to those of skill in the art. Typically, an immunogen, preferably a purified protein, is mixed with an adjuvant and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the

IL-174 protein or peptide of interest. For example, when appropriately high titers of antibody to the immunogen are obtained, usually after repeated immunizations, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be performed, if desired. See, e.g., Harlow and Lane Antibodies, A Laboratory Manual CSH Press, NY; or Coligan (ed.) Current Protocols in Immunology. Immunization can also be performed through other methods, e.g., DNA vector immunization. See, e.g., Wang, et al. (1997) Virology 228:278-284.

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Typically, following immunizations, spleen cells from the animal are immortalized, commonly by fusion with a myeloma cell. See, Kohler and Milstein (1976) Eur. J. Immunol. 6:511-519. Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods known in the art. See, e.g., Doyle, et al. (eds. 1994 and periodic supplements) Cell and Tissue Culture: Laboratory Procedures, Wiley, NY, NY. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according, e.g., to the general protocol outlined by Huse, et al. (1989) Science 246:1275-1281.

Antibodies or binding compositions, including binding fragments and single chain versions, against predetermined fragments of IL-174 polypeptides can be raised by immunization of animals with conjugates of the fragments with carrier proteins as described above. Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies can be screened for binding to normal or defective IL-174 protein. These monoclonal antibodies will usually bind with at least a  $K_D$  of about 1 mM, more usually at least about 300  $\mu$ M, typically at least about 10  $\mu$ M, more typically at least about 30  $\mu$ M, preferably at least about 10  $\mu$ M, and more preferably at least about 3  $\mu$ M or better.

In some instances, it is desirable to prepare monoclonal antibodies (mAbs) from various mammalian hosts, such as mice, rodents, primates, humans, etc. Description of techniques for preparing such monoclonal antibodies may be found in, e.g., Stites, et al. (eds.) Basic and Clinical Immunology (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane (1988) Antibodies: A Laboratory Manual CSH Press; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, New York, NY; and particularly in Kohler and Milstein (1975) Nature

256:495-497, which discusses one method of generating monoclonal antibodies.

Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are then fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing in vitro. The

5 population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

10 Other suitable techniques involve selection of libraries of antibodies in phage or similar vectors. See, e.g., Huse, et al. (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281; and Ward, et al. (1989) Nature 341:544-546. The polypeptides and antibodies of the present invention may be used with or without modification, including chimeric or humanized antibodies.

15 Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 20 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced, see, Cabilly, U.S. Patent No. 4,816,567; and Queen, et al. (1989) Proc. Nat'l Acad. Sci. USA 86:10029-10033; or made in transgenic mice, see Mendez, et al. (1997) Nature Genetics 15:146-156; Abgenix and Medarex technologies.

25 Antibody binding compounds, including binding fragments, of this invention can have significant diagnostic or therapeutic value. They can be useful as non-neutralizing binding compounds and can be coupled to toxins or radionuclides so that when the binding compound binds to the antigen, a cell expressing it, e.g., on its surface, is killed. Further, these binding compounds can be conjugated to drugs or other therapeutic agents, either 30 directly or indirectly by means of a linker, and may effect drug targeting.

### C. Other Molecules

Antibodies are merely one form of specific binding compositions. Other binding compositions, which will often have similar uses, include molecules that bind with specificity to IL-174, e.g., in a protein-binding partner fashion, an antibody-antigen interaction, or in a natural physiologically relevant protein-protein interaction, either 35 covalent or non-covalent, e.g., proteins which specifically associate with IL-174 protein.

The molecule may be a polymer, or chemical reagent. A functional analog may be a protein with structural modifications, or may be a structurally unrelated molecule, e.g., which has a molecular shape which interacts with the appropriate binding determinants.

Drug screening using antibodies or IL-174 or fragments thereof can be performed to identify compounds having binding affinity to IL-174, or can block or simulate the natural interaction with ligand. Subsequent biological assays can then be utilized to determine if the compound has intrinsic blocking activity and is therefore an antagonist. Likewise, a compound having intrinsic stimulating activity can signal to the cells via the IL-174 pathway and is thus an agonist in that it simulates the activity of a ligand. Mutein antagonists may be developed which maintain receptor binding but lack signaling.

Structural studies of the ligands will lead to design of new variants, particularly analogs exhibiting agonist or antagonist properties on the receptor. This can be combined with previously described screening methods to isolate muteins exhibiting desired spectra of activities.

As receptor specific binding molecules are provided, also included are small molecules identified by screening procedures. In particular, it is well known in the art how to screen for small molecules which interfere, e.g., with ligand binding to the receptor, often by specific binding to the receptor and blocking of binding by natural ligand. See, e.g., meetings on High Throughput Screening, International Business Communications, Southborough, MA 01772-1749. Such molecules may compete with natural ligands, and selectively bind to the IL-174.

### III. Immunoassays

Immunoassays are valuable in diagnosing a disease or disorder associated with IL-174 imbalance or pathology. Qualitative or quantitative measurement of a particular protein can be performed by a variety of immunoassay methods. For a review of immunological and immunoassay procedures in general, see Stites and Terr (eds.) (1991) Basic and Clinical Immunology (7th ed.). Moreover, the immunoassays of the present invention can be performed in many configurations, which are reviewed extensively in Maggio (ed. 1980) Enzyme Immunoassay CRC Press, Boca Raton, Florida; Tijan (1985) "Practice and Theory of Enzyme Immunoassays," Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publishers B.V., Amsterdam; and Harlow and Lane Antibodies: A Laboratory Manual, supra. See also Chan (ed. 1987) Immunoassay: A Practical Guide Academic Press, Orlando, FL; Price and Newman (eds. 1991) Principles and Practice of Immunoassays Stockton Press, NY; and Ngo (ed. 1988) Non-isotopic Immunoassays Plenum Press, NY.

Immunoassays for measurement of IL-174 proteins or peptides can be performed by a variety of methods known to those skilled in the art. In brief, immunoassays to measure the protein can be either competitive or noncompetitive binding assays. In competitive binding assays, the sample to be analyzed competes with a labeled analyte for specific binding sites on a capture agent bound to a solid surface. Preferably the capture agent is an antibody specifically reactive with IL-174 proteins produced as described above. The concentration of labeled analyte bound to the capture agent is inversely proportional to the amount of free analyte present in the sample.

In a competitive binding immunoassay, the IL-174 protein present in the sample 10 competes with labeled protein for binding to a specific binding agent, e.g., an antibody specifically reactive with IL-174 protein. The binding agent may be bound to a solid surface to effect separation of bound labeled protein from the unbound labeled protein. Alternatively, the competitive binding assay may be conducted in liquid phase and a variety 15 of techniques known in the art may be used to separate the bound labeled protein from the unbound labeled protein. Following separation, the amount of bound labeled protein is determined. The amount of protein present in the sample is inversely proportional to the amount of labeled protein binding.

Alternatively, a homogeneous immunoassay may be performed in which a separation step is not needed. In these immunoassays, the label on the protein is altered by 20 the binding of the protein to its specific binding agent. This alteration in the labeled protein results in a decrease or increase in the signal emitted by label, so that measurement of the label at the end of the immunoassay allows for detection or quantitation of the protein.

IL-174 proteins may also be determined by a variety of noncompetitive 25 immunoassay methods. For example, a two-site, solid phase sandwich immunoassay may be used. In this type of assay, a binding agent for the protein, for example an antibody, is attached to a solid support. A second protein binding agent, which may also be an antibody, and which binds the protein at a different site, is labeled. After binding at both sites on the protein has occurred, the unbound labeled binding agent is removed and the amount of labeled binding agent bound to the solid phase is measured. The amount of 30 labeled binding agent bound is directly proportional to the amount of protein in the sample.

Western blot analysis can be used to determine the presence of IL-174 proteins in a sample. Electrophoresis is carried out, e.g., on a tissue sample suspected of containing the protein. Following electrophoresis to separate the proteins, and transfer of the proteins to a suitable solid support, e.g., a nitrocellulose filter, the solid support is incubated with an 35 antibody reactive with the protein. This antibody may be labeled, or alternatively may be

detected by subsequent incubation with a second labeled antibody that binds the primary antibody.

The immunoassay formats described above may employ labeled assay components. The label may be coupled directly or indirectly to the desired component of the assay 5 according to methods well known in the art. A wide variety of labels and methods may be used. Traditionally, a radioactive label incorporating  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , or  $^{32}\text{P}$  was used. Non-radioactive labels include ligands which bind to labeled antibodies, fluorophores, chemiluminescent agents, enzymes, and antibodies which can serve as specific binding pair 10 members for a labeled ligand. The choice of label depends on sensitivity required, ease of conjugation with the compound, stability requirements, and available instrumentation. For a review of various labeling or signal producing systems which may be used, see U.S. Patent No. 4,391,904.

Antibodies reactive with a particular protein can also be measured by a variety of immunoassay methods. Thus modifications of the above procedures may be used to 15 determine the amounts or affinities of various IL-174 antibodies or antibody preparations. For a review of immunological and immunoassay procedures applicable to the measurement of antibodies by immunoassay techniques, see Stites and Terr (eds.) Basic and Clinical Immunology (7th ed.) *supra*; Maggio (ed.) Enzyme Immunoassay, *supra*; and Harlow and Lane Antibodies. A Laboratory Manual, *supra*.

20 Screens to evaluate the binding and activity of mAbs and binding compositions encompass a variety of methods. Binding can be assayed by detectably labeling the antibody or binding composition as described above. Cells responsive to IL-174 can be used to assay antibody or binding composition.

Evaluation of antibodies can be performed in other animals, e.g., humans using 25 various methods. For example, blood samples are withdrawn from patients suffering from an indicated disease or disorder before and after treatment, e.g., with a candidate mAb.

#### IV. Uses

IL-174 now has demonstrated effects on various cells, which effects may be 30 indirect, as well as direct. A statistically significant change in the numbers or effects on cells will typically be at least about 10%, preferably 20%, 30%, 50%, 70%, 90%, or more. Effects of greater than 100%, e.g., 130%, 150%, 2X, 3X, 5X, etc., will often be desired. The effects may be specific in reducing symptoms or signs of the indicated conditions.

The present invention is useful in the treatment of medical conditions or diseases 35 associated with immunological conditions described. See, e.g., Frank, et al. (eds. 1995) Samter's Immunologic Diseases, 5th Ed., vols. I-II, Little, Brown and Co., Boston, MA.

The agonists or antagonists described may be combined with other treatments of the medical conditions described herein, e.g., another cytokine involved in Th1/Th2 balance (such as IL-1 $\gamma$  and/or IL-12 to Th1; or IL-4 to Th2), an antibiotic, immune suppressive therapeutic, immune adjuvant, analgesic, anti-inflammatory drug, growth factor, 5 vasodilator, or vasoconstrictor.

Preferred combination therapies include IL-174 agonists with various therapeutics which promote Th2 type immune responses (e.g., IL-4, IL-1 $\gamma$  antagonists, IL-12 antagonists), inflammatory agents, such as topical, transdermal, or systemic irritants, various growth factors, and hematopoietic factors. Antagonists of IL-174, e.g., antibodies 10 or ligand muteins, can be combined with therapeutics which promote Th1 type immune responses (e.g., IL-1 $\gamma$  and/or IL-12, IL-4 antagonists), or anti-inflammatories (e.g., steroids, glucocortical steroids).

Standard immunological techniques are described, e.g., in Hertzenberg, et al. (eds. 1996) Weir's Handbook of Experimental Immunology vols. 1-4, Blackwell Science; 15 Coligan (1991) Current Protocols in Immunology Wiley/Greene, NY; and Methods in Enzymology volumes 70, 73, 74, 84, 92, 93, 108, 116, 121, 132, 150, 162, and 163. These will allow use of the reagents for purifying cell subpopulations, etc.

To prepare pharmaceutical or sterile compositions including IL-174, antagonists, or combinations, the reagent is admixed with a pharmaceutically acceptable carrier or 20 excipient which is preferably inert. Preparation of such pharmaceutical compositions is known in the art, see, e.g., Remington's Pharmaceutical Sciences and U.S. Pharmacopeia: National Formulary, Mack Publishing Company, Easton, PA (1984).

Agonists, e.g., natural ligand, or antagonists, e.g., antibodies or binding 25 compositions, are normally administered parenterally, preferably intravenously. Since such protein or peptide antagonists may be immunogenic they are preferably administered slowly, either by a conventional IV administration set or from a subcutaneous depot, e.g. as taught by Tomasi, et al., U.S. patent 4,732,863.

When administered parenterally the therapeutics will often be formulated in a unit 30 dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are typically inherently nontoxic and nontherapeutic. Agonists, which typically will be smaller biologics, will typically be administered in smaller doses than antibody biologics. The antagonist may be administered in aqueous vehicles such as water, saline or buffered vehicles with or without various additives and/or diluting agents. Alternatively, a suspension, such as a zinc 35 suspension, can be prepared to include the peptide. Such a suspension can be useful for subcutaneous (SQ), intradermal (ID), or intramuscular (IM) injection. The proportion of

therapeutic entity and additive can be varied over a broad range so long as both are present in effective amounts. The therapeutic is preferably formulated in purified form substantially free of aggregates, other proteins, endotoxins, and the like, at concentrations of about 5 to 30 mg/ml, preferably 10 to 20 mg/ml. Preferably, the endotoxin levels are less than 2.5 EU/ml. See, e.g., Avis, et al. (eds. 1993) Pharmaceutical Dosage Forms: Parenteral Medications 2d ed., Dekker, NY; Lieberman, et al. (eds. 1990) Pharmaceutical Dosage Forms: Tablets 2d ed., Dekker, NY; Lieberman, et al. (eds. 1990) Pharmaceutical Dosage Forms: Disperse Systems Dekker, NY; Fodor, et al. (1991) Science 251:767-773; Coligan (ed.) Current Protocols in Immunology; Hood, et al. Immunology Benjamin/Cummings; 5 Paul (ed. 1997) Fundamental Immunology 4th ed., Academic Press; Parce, et al. (1989) Science 246:243-247; Owicki, et al. (1990) Proc. Nat'l Acad. Sci. USA 87:4007-4011; and 10 Blundell and Johnson (1976) Protein Crystallography, Academic Press, New York.

15 Selecting an administration regimen for a therapeutic agonist or antagonist depends on several factors, including the serum or tissue turnover rate of the therapeutic, the immunogenicity of the therapeutic, or the accessibility of the target cells. Preferably, an administration regimen maximizes the amount of therapeutic delivered to the patient consistent with an acceptable level of side effects. Accordingly, the amount of therapeutic delivered depends in part on the particular agonist or antagonist and the severity of the condition being treated. Guidance in selecting appropriate doses of antibodies is found in 20 the literature on therapeutic uses, e.g. Bach et al., chapter 22, in Ferrone, et al. (eds. 1985), Handbook of Monoclonal Antibodies Noges Publications, Park Ridge, NJ; and Russell, pgs. 303-357, and Smith et al., pgs. 365-389, in Haber, et al. (eds. 1977) Antibodies in Human Diagnosis and Therapy Raven Press, New York, NY.

25 Determination of the appropriate dose is made by the clinician, e.g., using parameters or factors known in the art to affect treatment or predicted to affect treatment. Generally, the dose begins with an amount somewhat less than the optimum dose and it is increased by small increments thereafter until the desired or optimum effect is achieved relative to any negative side effects. Preferably, an antibody or binding composition thereof that will be used is derived from the same species as the animal targeted for treatment, 30 thereby minimizing a humoral response to the reagent.

35 The total weekly dose ranges for antibodies or fragments thereof, which specifically bind to IL-174, range generally from about 1 ng, more generally from about 10 ng, typically from about 100 ng; more typically from about 1  $\mu$ g, more typically from about 10  $\mu$ g, preferably from about 100  $\mu$ g, and more preferably from about 1 mg per kilogram body weight. Although higher amounts may be more efficacious, the lower doses typically will

have fewer adverse effects. Generally the range will be less than 100 mg, preferably less than about 50 mg, and more preferably less than about 25 mg per kilogram body weight.

5 The weekly dose ranges for antagonists, e.g., antibody, binding fragments, range from about 10  $\mu$ g, preferably at least about 50  $\mu$ g, and more preferably at least about 100  $\mu$ g per kilogram of body weight. Generally, the range will be less than about 1000  $\mu$ g, preferably less than about 500  $\mu$ g, and more preferably less than about 100  $\mu$ g per kilogram of body weight. Dosages are on a schedule which effects the desired treatment and can be periodic over shorter or longer term. In general, ranges will be from at least about 10  $\mu$ g to about 50 mg, preferably about 100  $\mu$ g to about 10 mg per kilogram body weight.

10 Agonists, or other antagonists of the ligands, e.g., muteins, are also contemplated. Hourly dose ranges for cytokine or muteins range from at least about 10  $\mu$ g, generally at least about 50  $\mu$ g, typically at least about 100  $\mu$ g, and preferably at least 500  $\mu$ g per hour. Generally the dosage will be less than about 100 mg, typically less than about 30 mg, preferably less than about 10 mg, and more preferably less than about 6 mg per hour.

15 General ranges will be from at least about 1  $\mu$ g to about 1000  $\mu$ g, preferably about 10  $\mu$ g to about 500  $\mu$ g per hour.

20 The present invention also provides for administration of IL-174 antibodies or binding compositions in combination with known therapies, e.g., therapeutics which alleviate the symptoms associated with excessive inflammatory responses, such as steroids, particularly glucocorticoids. Daily dosages for glucocorticoids will range from at least about 1 mg, generally at least about 2 mg, and preferably at least about 5 mg per day. Generally, the dosage will be less than about 100 mg, typically less than about 50 mg, preferably less than about 20 mg, and more preferably at least about 10 mg per day. In general, the ranges will be from at least about 1 mg to about 100 mg, preferably from about 25 2 mg to 50 mg per day.

25 The phrase "effective amount" means an amount sufficient to effect a desired response, or to ameliorate a symptom or sign of the indicated condition. Typical mammalian hosts will include mice, rats, cats, dogs, and primates, including humans. An effective amount for a particular patient may vary depending on factors such as the 30 condition being treated, the overall health of the patient, the method, route, and dose of administration and the severity of side affects. Preferably, the effect will result in a change in quantitation of at least about 10%, preferably at least 20%, 30%, 50%, 70%, or even 90% or more. When in combination, an effective amount is in ratio to a combination of components and the effect is not limited to individual components alone.

35 An effective amount of therapeutic will modulate the symptoms typically by at least about 10%; usually by at least about 20%; preferably at least about 30%; or more preferably

at least about 50%. Alternatively, modulation of movement will mean that the movement or trafficking of various cell types is affected. Such will result in, e.g., statistically significant and quantifiable changes in the numbers of cells being affected. This may be an increase or decrease in the numbers of target cells being attracted within a time period or 5 target area.

The present invention provides reagents which will find use in therapeutic applications as described elsewhere herein, e.g., in the general description for treating disorders associated with the indicated conditions. See, e.g., Berkow (ed.) The Merck Manual of Diagnosis and Therapy, Merck & Co., Rahway, N.J.; Thorn, et al. Harrison's Principles of Internal Medicine, McGraw-Hill, NY; Gilman, et al. (eds. 1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn; Langer (1990) Science 249:1527-1533; and Merck Index, Merck & Co., Rahway, New Jersey.

15 Antibodies to IL-174 proteins may be used for the identification or sorting of cell populations expressing IL-174 protein. Methods to sort such populations are well known in the art, see, e.g., Melamed, et al. (1990) Flow Cytometry and Sorting Wiley-Liss, Inc., New York, NY; Shapiro (1988) Practical Flow Cytometry Liss, New York, NY; and Robinson, et al. (1993) Handbook of Flow Cytometry Methods Wiley-Liss, New York, NY.

20 Populations of cells expressing the IL-174 receptor can also be purified using magnetic beads as described, e.g., in Bieva, et al. (1989) Exp. Hematol. 17:914-920; Hernebtub, et al. (1990) Bioconj. Chem. 1:411-418; Vaccaro (1990) Am. Biotechnol. Lab. 3:30.

25 Moreover, antisense nucleic acids may be used. For example, antisense constructs specific to nucleic acids encoding, e.g., the ligand, may function in a manner like ligand antagonists, and antisense constructs specific to those encoding the receptor may function like receptor antagonists. See, e.g., Stepkowski, et al. (1998) Transplantation 66:699-707; and ISIS Pharmaceuticals technology. Thus, it may be possible to block the signaling through these pathways with antisense nucleic acids.

30 Using the assay methods described above, the antibodies or binding compositions are useful in diagnosing disease states which result in the indicated disorders. Antibodies raised against an IL-174 protein will also be useful to raise anti-idiotypic antibodies. These will be useful in detecting or diagnosing various immunological conditions related to expression of the respective antigens. Combinations of these signals may be also pursued.

35 The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the inventions to the specific embodiments.

## EXAMPLES

## I. General Methods

The cytokine IL-174, from mouse and human, has been described earlier in PCT/US00/00006, which is incorporated herein by reference for all purposes.

5 Some of the standard methods are described or referenced, e.g., in Maniatis, et al. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor Press; Sambrook, et al. (1989) Molecular Cloning: A Laboratory Manual, (2d ed.), vols. 1-3, CSH Press, NY; Ausubel, et al., Biology, Greene Publishing Associates, Brooklyn, NY; or Ausubel, et al. (1987 and Supplements) Current Protocols in Molecular Biology, Greene/Wiley, New York; Innis, et al. (eds.) (1990) PCR Protocols: A Guide to Methods and Applications Academic Press, N.Y. Methods for protein purification include such methods as ammonium sulfate precipitation, column chromatography, electrophoresis, centrifugation, crystallization, and others. See, e.g., Ausubel, et al. (1987 and periodic supplements); Deutscher (1990) "Guide to Protein Purification" in Methods in Enzymology, vol. 182, and other volumes in this series; manufacturer's literature on use of protein purification products, e.g., Pharmacia, Piscataway, N.J., or Bio-Rad, Richmond, CA; and Coligan, et al. (eds.) (1995 and periodic supplements) Current Protocols in Protein Science, John Wiley & Sons, New York, NY. Combination with recombinant techniques allow fusion to appropriate segments, e.g., to a FLAG sequence or an equivalent which can be fused via a protease-removable sequence. See, e.g., Hochuli (1989) Chemische Industrie 12:69-70; Hochuli (1990) "Purification of Recombinant Proteins with Metal Chelate Absorbent" in Setlow (ed.) Genetic Engineering, Principle and Methods 12:87-98, Plenum Press, N.Y.; and Crowe, et al. (1992) QIAexpress: The High Level Expression & Protein Purification System QIAGEN, Inc., Chatsworth, CA.

10 15 20 25

Standard immunological techniques are described, e.g., in Hertzenberg, et al. (eds. 1996) Weir's Handbook of Experimental Immunology vols. 1-4, Blackwell Science; Coligan (1991) Current Protocols in Immunology Wiley/Greene, NY; and Methods in Enzymology vols. 70, 73, 74, 84, 92, 93, 108, 116, 121, 132, 150, 162, and 163.

30 35

FACS analyses are described in Melamed, et al. (1990) Flow Cytometry and Sorting Wiley-Liss, Inc., New York, NY; Shapiro (1988) Practical Flow Cytometry Liss, New York, NY; and Robinson, et al. (1993) Handbook of Flow Cytometry Methods Wiley-Liss, New York, NY.

## II. Antibody Production

Appropriate mammals are immunized with appropriate amounts of IL-174 gene transfected cells, e.g., intraperitoneally every 2 weeks for 8 weeks. Typically, rodents are

used, though other species should accommodate production of selective and specific antibodies. The final immunization is given intravenously (IV) through the tail vein.

Generic polyclonal antibodies may be collected. Alternatively, monoclonal antibodies can be produced. For example, four days after the IV injection, the spleen is removed and fused to SP2/0 and NS1 cells. HAT resistant hybridomas are selected, e.g., using a protocol designed by Stem Cell Technologies (Vancouver, BC). After 10 days of HAT selection, resistant foci are transferred to 96 well plates and expanded for 3 days. Antibody containing supernatants are analyzed, e.g., by FACS for binding to NIH3T3/surface IL-174 transfectants. Many different IL-174 mAbs are typically produced. Those antibodies may be isolated and modified, e.g., by labeling or other means as is standard in the art. See, e.g., Harlow and Lane (1988) Antibodies: A Laboratory Manual CSH Press; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, New York, NY. Methods to conjugate magnetic reagents, toxic entities, labels, attach the antibodies to solid substrates, to sterile filter, etc., are known in the art.

15

### III. IL-174 Antagonists

Various antagonists of IL-174 are made available. For example, antibodies against the cytokine itself may block the binding of ligand to its receptor, thereby serving as a direct receptor antagonist. Other antagonists may function by blocking the binding of ligand to receptor, e.g., by binding to the ligand in a way to preclude the possibility of binding to the receptor. Other antagonists, e.g., mutein antagonists, may bind to the receptor without signaling, thereby blocking a true agonist from binding. Many of these may serve to block the signal transmitted to target cells, specifically IL-174 responsive cells.

Information on the criticality of particular residues is determined using standard procedures and analysis. Standard mutagenesis analysis is performed, e.g., by generating many different variants at determined positions, e.g., at the positions identified above, and evaluating biological activities of the variants. This may be performed to the extent of determining positions which modify activity, or to focus on specific positions to determine the residues which can be substituted to either retain, block, or modulate biological activity.

Alternatively, analysis of natural variants can indicate what positions tolerate natural mutations. This may result from populational analysis of variation among individuals, or across strains or species. Samples from selected individuals are analyzed, e.g., by PCR analysis and sequencing. This allows evaluation of population polymorphisms.

35 IV. Adenoviral infection

Adenovirus constructs were prepared. Mice were infected with adenovirus-IL-74 via the intranasal route or the intravenous route. See, e.g., Hitt, et al. (1997) Adv. Pharmacol. 40:137-195. The doses of adenovirus-IL-74 given ranged from  $5 \times 10^9$  to  $5 \times 10^{10}$  particles. Mice were evaluated 7, 14, 21, and 35 days post infection using standard 5 histologic, immunologic, and hematologic methods

#### V. Histologic analysis

Tissues from mice were fixed in formalin, routinely processed, sectioned, and stained with hematoxylin and eosin for microscopic examination. The organs examined 10 included the lung, heart, liver, kidney, spleen, bone marrow, and gastrointestinal tract. See, e.g., Kerr (1999) Atlas of Functional Histology; Sternberg (ed. 1998) Histology for Pathologists; and Stevens and Lowe (1996) Human Histology.

#### VI. Hematopoietic colony forming assays

15 Hematopoietic progenitors in the spleens of mice were enumerated in routine colony-forming assays. Spleen cells were depleted of erythrocytes by hypotonic lysis. Spleen cells were incubated with unmodified rat monoclonal antibodies specific for CD2 (RM2.2; see Yagata, et al. (1989) Proc. Nat'l Acad. Sci. USA 86:645-649), CD8 (53.6.7; see Ledbetter, et al. Immunol. Rev. 47:63-90), B220 (RA3-6B2; see Coffman, et al. (1981) Nature 289:681-683), Mac1 (M1/70; see Springer, et al. (1979) Eur. J. Immunol. 9:301-306), GR1 (RB6-8C5; see Julia, et al. (1988) Eur. J. Immunol. 19:1819-1826), and erythrocytes (Ter-119; see Spangrude, et al. (1990) Exp. Hemat. 18:920-926). The lineage 20 positive cells depleted by negative selection using goat anti-rat coated BioMagR beads (PerSeptive Biosystems, Framingham, MA) in two successive rounds of treatment. The remaining lineage negative spleen cells were seeded in 35 mm culture dishes containing 1 25 ml modified Iscove's medium (JRH, Kansas City, KS), 20% fetal calf serum (FCS) (JRH) 50 mM 2-mercaptoethanol, and 0.8% (wt/vol) methylcellulose. All cultures were supplemented with saturating concentrations of various growth factors. Cultures were 30 incubated at 37° C in a humidified atmosphere flushed with 5% CO<sub>2</sub>. After 10 days of incubation, colonies were analyzed for number and size. Cell morphologies were determined after colonies were applied to glass slides and stained with Wright's Giemsa for microscopic examination. See Metcalf (1984) The Hemopoietic Colony Stimulating Factors, Elsevier, Amsterdam.

35 VII. Blood cell analysis

Blood from mice was collected in Isotonic Buffered Diluent (BioChem ImmunoSystems, Allentown, PA) and hemoglobin, hematocrit, white blood cell, red blood cell, and platelet counts were determined by an automatic cell counter (Serono 9010, Serono-Baker Diagnostics, Inc., Allentown, PA). Blood smears were stained with Wright's Giemsa and differential counts were performed with the aid of a microscope. See, e.g., 5 Dainiak (1990) The Biology of Hematopoiesis, Wiley-Liss Inc., New York; Testa and Molineux (1993) Haemopoiesis, Oxford University Press, New York.

#### VIII. Determination of Antibody Isotypes

Sera from mice were serially diluted in isotonic buffer in microtiter wells coated 10 with rat anti-mouse heavy-chain class-specific antibodies (anti- $\alpha$ , - $\gamma$ 1, - $\gamma$ 2a, and - $\epsilon$ ). A biotinylated secondary antibody (specific for heavy-chain classes) was added and then reacted with streptavidin-HRP (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The colored product produced by this TMB peroxidase substrate system (Kirkegaard 15 & Perry Laboratories, Gaithersburg, MD) is detected under a long-wavelength UV lamp (450 nanometers). See, e.g., Coligan, et al. (eds. 1991 and supplements) Current Protocols in Immunology, Greene/Wiley.

#### IX. Bronchio-alveolar lavage fluid analysis

Bronchio-alveolar lavage (BAL) fluid was collected from sacrificed mice by 20 inserting a needle into the trachea and dispensing 1 ml of DMEM media. 200  $\mu$ l of the recovered sample was examined for cell composition by cytopsin followed by Wright-Giemsa stain. Differential counts were performed with the aid of a microscope.

#### 25 X. Quantification of Gene Expression by Real-Time RT-PCR

Sample tissues were excised from mice following treatment and were snap-frozen in liquid nitrogen. Total RNA was extracted using RNAstat60 (Molecular Research Center) according to the manufacturer's directions and were stored at -80° C in nuclease-free. For 30 cDNA synthesis RNA was incubated with 10 units of DNase I (Boehringer Mannheim) in the presence of RNasin (Promega) for 30 minutes at 37° C. The samples were then heat inactivated at 95° C for 10 minutes, chilled, and reverse transcribed with Superscript II 35 reverse transcriptase (Gibco/BRL) with random hexamers according to the manufacturer's protocol. Primers were either obtained from Perkin Elmer or generated with Primer Express software (Perkin Elmer) and were synthesized in the DNAX primer core facility. Whenever possible, primer pairs were designed to span intron/exon borders. Samples were then subjected to 40 cycles of amplification of 95° C for 15 seconds followed by 60° C for 1

minute using an ABI GeneAmp 5700 sequence detection system and SYBR green buffer according to the manufacturer (Perkin Elmer). PCR amplification of the housekeeping gene ubiquitin was performed for each sample to control for sample loading and to allow normalization between samples according to the manufacturer's instructions (Perkin Elmer).

5 Both water and genomic DNA controls were included to insure specificity. Each data point was examined for integrity by analysis of the amplification plot and disassociation curves.

10 All citations herein are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

15 Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled; and the invention is not to be limited by the specific embodiments that have been presented herein by way of example.